

University of Groningen

## The role of *Hansenula polymorpha* MIG1 homologues in catabolite repression and pexophagy

Stasyk, Olena G.; Van Zutphen, Tim; Kang, Huyn Ah; Stasyk, Oleh V.; Veenhuis, Marten; Sibirny, Andriy A.

*Published in:*  
Fems Yeast Research

*DOI:*  
[10.1111/j.1567-1364.2007.00286.x](https://doi.org/10.1111/j.1567-1364.2007.00286.x)

**IMPORTANT NOTE:** You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2007

[Link to publication in University of Groningen/UMCG research database](#)

### *Citation for published version (APA):*

Stasyk, O. G., Van Zutphen, T., Kang, H. A., Stasyk, O. V., Veenhuis, M., & Sibirny, A. A. (2007). The role of *Hansenula polymorpha* MIG1 homologues in catabolite repression and pexophagy. *Fems Yeast Research*, 7(7), 1103-1113. <https://doi.org/10.1111/j.1567-1364.2007.00286.x>

### Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

### Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

*Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.*

## RESEARCH ARTICLE

# The role of *Hansenula polymorpha* MIG1 homologues in catabolite repression and pexophagy

Olena G. Stasyk<sup>1</sup>, Tim van Zutphen<sup>2</sup>, Huyn Ah Kang<sup>3</sup>, Oleh V. Stasyk<sup>1</sup>, Marten Veenhuis<sup>2</sup> & Andriy A. Sibirny<sup>1,4</sup>

<sup>1</sup>Institute of Cell Biology, National Academy of Sciences of Ukraine, Lviv, Ukraine; <sup>2</sup>Eukaryotic Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Biological Centre, Haren, The Netherlands; <sup>3</sup>Omics and Integration Research Center, Korea Research Institute of Bioscience and Biotechnology, Deajeon, Korea; and <sup>4</sup>Department of Metabolic Engineering, Rzeszów University, Rzeszów, Poland

**Correspondence:** Andriy A. Sibirny, Institute of Cell Biology, National Academy of Sciences of Ukraine, Drahomanov Street 14/16, Lviv 79005, Ukraine. Tel.: +380 32 2612163; fax: +380 32 2612148; e-mail: sibirny@cellbiol.lviv.ua

Received 15 February 2007; revised 7 June 2007; accepted 11 June 2007.  
First published online August 2007.

DOI:10.1111/j.1567-1364.2007.00286.x

Editor: Gerd Gellissen

## Keywords

yeast; *Hansenula polymorpha*; catabolite repression; pexophagy; transcriptional repressors.

## Abstract

In the methanol-utilizing yeast *Hansenula polymorpha*, glucose and ethanol trigger the repression of peroxisomal enzymes at the transcriptional level, and rapid and selective degradation of methanol-induced peroxisomes by means of a process termed pexophagy. In this report we demonstrate that deficiency in the putative *H. polymorpha* homologues of transcriptional repressors Mig1 (*HpMig1* and *HpMig2*), as well as *HpTup1*, partially and differentially affects the repression of peroxisomal alcohol oxidase by sugars and ethanol. As reported earlier, deficiency in *HpTup1* leads to impairment of glucose- or ethanol-induced macropexophagy. In *H. polymorpha* *mig1mig2* double-deletion cells, macropexophagy was also substantially impaired, whereas micropexophagy became a dominant mode of autophagic degradation. Our findings suggest that homologues of the elements of the *Saccharomyces cerevisiae* main repression pathway have pleiotropic functions in *H. polymorpha*.

## Introduction

It is well established that intact peroxisomes are indispensable for methylotrophic growth in yeasts. These organelles harbour key enzymes that catalyze the first steps of methanol utilization [as alcohol oxidase (AO), catalase, dihydroxyacetone synthase], and provide compartmentalization of toxic methanol catabolites, formaldehyde and hydrogen peroxide (Veenhuis *et al.*, 1983b; van der Klei *et al.*, 2006). Peroxisomes, however, are redundant for growth on rich carbon sources such as hexoses and disaccharides. When methanol-grown cells are shifted to sugar carbon substrates or ethanol, peroxisomes are rapidly and selectively degraded in vacuoles (Veenhuis *et al.*, 1983a), whereas genes of peroxisomal enzymes are subjected to tight transcriptional repression (Roggenkamp *et al.*, 1984). Such coordinated genetically programmed catabolite regulation ensures a fast and efficient cellular response to environmental changes.

It was originally assumed that the molecular pathway of glucose catabolite repression in methylotrophic yeasts may

mimic that of the conventional yeast *Saccharomyces cerevisiae* (the so-called Snf1-Mig1 pathway). Its main components, the lack of which abolishes repression signalling, are hexokinase II subunit, Snf1 protein kinase, and the Mig1 repressor that tethers or facilitates the binding of the Tup1-Ssn6 general repressor to promoters of repressible genes in the presence of glucose (Gancedo, 1998; Papamichos-Chronakis *et al.*, 2004). Several observations support this hypothesis. First, Mig1-Ssn6-Tup1 and the Snf1 complexes are highly conserved among different yeasts (Gancedo, 1998). Second, promoters of *H. polymorpha* alcohol oxidase (Pereira & Hollenberg, 1996) and maltase (Alamae *et al.*, 2003) genes were demonstrated to be repressed by glucose in *S. cerevisiae*, suggesting that they are targets of the host's repression pathway. Recently, hexose phosphorylation activity was shown to be essential for repression signalling in *H. polymorpha*: hexokinase mutant was insensitive to fructose (but not glucose) repression, whereas in double hexo- and glucokinase mutant, glucose repression was also abrogated (Kramarenko *et al.*, 2000). The above findings suggested

that glucose-repressible genes of C-1 metabolism in *H. polymorpha* may be the target of Mig1/(Tup1-Ssn6)-mediated transcriptional repression, analogous to the case of baker's yeast. However, Oliveira *et al.* (2003) reported that HpTup1 is not essential for glucose repression of peroxisomal enzymes in *H. polymorpha*.

Adding to the emerging complexity of repression pathway in this yeast, we recently identified a hexose transporter homologue, HpGcr1, which is most probably involved in the first stages of glucose (but not of sucrose or ethanol) signalling for repression, but may also interfere with high-affinity glucose transport (Stasyk *et al.*, 2004). Its close homologues in *S. cerevisiae*, the putative glucose sensors Snf3 and Rgt2, were not implicated in the repression mechanism, but instead differentially regulate the induction of functional hexose transporters in response to extracellular glucose (Johnston & Kim, 2005). It has to be emphasized that, in *H. polymorpha* and other methylotrophs, disaccharides and ethanol are potent repressors of gene expression involved in C-1 metabolism (Sibirny *et al.*, 1988; Stasyk *et al.*, 2004). However, nothing is known of the specific molecular components of ethanol- or disaccharide-triggered repression, whether different carbon source-dependent pathways physically converge, and, if so, at what stage they do so.

With regard to pexophagy, significant progress has been achieved in recent years in understanding its molecular mechanisms and regulation, with *H. polymorpha* being a very productive model organism, as detailed in recent reviews (Dunn *et al.*, 2005; Monastyrska & Klionsky, 2006; Sakai *et al.*, 2006).

Two morphologically distinguishable pexophagy mechanisms have been observed in *H. polymorpha*: selective macropexophagy-triggered by a shift in carbon source (i.e. from methanol to glucose or ethanol) (Veenhuis *et al.*, 1983a; Tuttle *et al.*, 1993), or cold shock (Komduur *et al.*, 2004); and nonselective micropexophagy triggered upon nitrogen starvation, leading to bulk turnover by general autophagy (Monastyrska *et al.*, 2002). Most ATG (AuTop-haGy-related) genes involved in pexophagy identified so far in *H. polymorpha* and other yeasts are conserved among eukaryotes and also appear to affect general autophagy. Those essential only for pexophagy are in many cases organism-specific (Meijer *et al.*, 2007). The latter include *H. polymorpha* ATG11 and ATG25 (Monastyrska *et al.*, 2005), ATG26 (our unpublished results), and TUP1 (Leao-Helder *et al.*, 2004). HpTup1 is a first representative of transcriptional repressors involved in pexophagy. As macropexophagy, in contrast to nitrogen starvation-induced microautophagy, is insensitive to cycloheximide (CHX) (i.e. independent of protein synthesis *de novo*) (Monastyrska *et al.*, 2005), the function of HpTup1 in pexophagy has been suggested to be indirect (Leao-Helder *et al.*, 2004).

Knowledge of the molecular mechanisms of catabolite regulation in *H. polymorpha* is of basic and practical interest. Mutants with altered catabolite regulation of the AO gene promoter may be promising hosts for the expression of recombinant proteins in methanol-free media (Krasovska *et al.*, 2007), and protein products can be targeted to peroxisomes applying this organelle as a storage vesicle (van Dijk *et al.*, 2000). The availability of the full *H. polymorpha* genome sequence (Ramezani-Rad *et al.*, 2003) made it possible to search for orthologues of *S. cerevisiae* genes involved in catabolite repression and to study their function. In this report we analyse the possible role of two *H. polymorpha* Mig1 homologues (designated HpMig1 and HpMig2) in the processes of catabolite repression and pexophagy.

## Materials and methods

### Microorganisms and growth conditions

The following *H. polymorpha* strains were used in this study: NCYC495 *leu1-1* (Gleeson & Sudbery, 1988),  $\Delta mig1$  deletion mutant,  $\Delta mig2$  deletion mutant,  $\Delta mig1\Delta mig2$  double-deletion mutant (all this study),  $\Delta tup1 leu1-1$  (Leao-Helder *et al.*, 2004),  $\Delta gcr1$  (Stasyk *et al.*, 2004).

*Hansenula polymorpha* cells were grown at 37 °C in rich YPD medium (1% peptone, 2% yeast extract, 1% glucose) or minimal media containing 0.67% yeast nitrogen base (YNB, Difco, Detroit, MI) supplemented with 1% glucose (YND), 1% sucrose (YNS), 1% ethanol (YNE), or 1% methanol (YNM), unless stated otherwise. For auxotrophic strains, leucine (40 mg L<sup>-1</sup>) was added. For the selection of yeast transformants via geneticine (G418) resistance, 0.4 g L<sup>-1</sup> of G418 was added to YPD plates.

The *Escherichia coli* DH5 $\alpha$  strain was used as a host for propagation of plasmids and grown at 37 °C in Luria-Bertani (LB) medium as described in Sambrook *et al.* (1989).

### Molecular-biology techniques

Standard cloning and DNA manipulation techniques were applied (Sambrook *et al.*, 1989). Genomic DNA of *H. polymorpha* was isolated using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Restriction endonucleases and DNA ligase (Fermentas, Vilnius, Lithuania) were used according to the manufacturer's specifications. PCR-amplification of the fragments of interest was performed with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) according to the manufacturer's specification. The primers used in this study are listed in Table 1. The transformation of *H. polymorpha* by electroporation was carried out as described previously (Faber *et al.*, 1994).

**Table 1.** Primers used in this study

Gene specificity	Name of primer	Sequence
<i>HpMIG1</i>	OL54	5'-TGTGGATCCTTTTTTGGCGTGATGT-3'
<i>HpMIG1</i>	OL55	5'-GGTCTAGATCACTCAGAAAACATGG-3'
<i>HpMIG1</i>	OL57	5'-TGGAAGCTTAGAAAGACCTGCTTGCTG-3'
<i>HpMIG1</i>	Mi12Nf	5'-CCCAAGCTTGGTGTAGTTGTCGGAG-3'
<i>HpMIG1</i>	Mi12Nr	5'-ATAAGAATGCGGCCCTACACTGTAGCTACG-3'
<i>HpMIG1</i>	Mi12Cf	5'-CGAGACCTGCATACATG-3'
<i>HpMIG1</i>	Mi12Cr	5'-CCGGAATTCGCATCCAGAGTACTC-3'
<i>HpMIG2</i>	OL91	5'-AAACTAAACAGGGATCG-3'
<i>HpMIG2</i>	OL92	5'-AAAATACGACTGCAGCGA-3'
<i>HpMIG2</i>	OL153	5'-TGTGGATCCAAATTTTACACCTCAGG-3'
<i>HpMIG2</i>	OL154	5'-CCGATATCTAGAGACGTGCTTCCAT-3'
<i>HpMIG2</i>	OL155	5'-TCCATAGAAAGCTTCTGAACAGCCCCGAAGA-3'
<i>HpMIG2</i>	OL156	5'-GGAAGCTTGAGCTCGAAATATTTCTTCTTGC-3'
<i>HpLEU2</i>	OL143	5'-GCACAATGTTCTTACTCAT-3'
<i>ScLEU2</i>	CK15	5'-TGTAATTGTTGGGATTCC-3'

### Protein sequence analyses

For analysis of DNA and amino acid sequences, MACVECTOR software (IBI, New Haven, CT) was used. Sequence alignments and phylogenetic analysis were performed using the CLUSTALW version 1.6 algorithm (Corpet, 1988). The BLAST Network Service of the National Center for Biotechnology Information (Bethesda, MD; <http://www.ncbi.nlm.nih.gov/BLAST/>) was used to search for amino acid sequence similarities. For pattern and profile search, the PROSCAN program at [http://npsa-pbil.ibcp.fr/cgi-bin/pattern\\_prosite.pl](http://npsa-pbil.ibcp.fr/cgi-bin/pattern_prosite.pl) was used (Combet *et al.*, 2000).

### Construction of *HpMIG1* and *HpMIG2* deletion strains

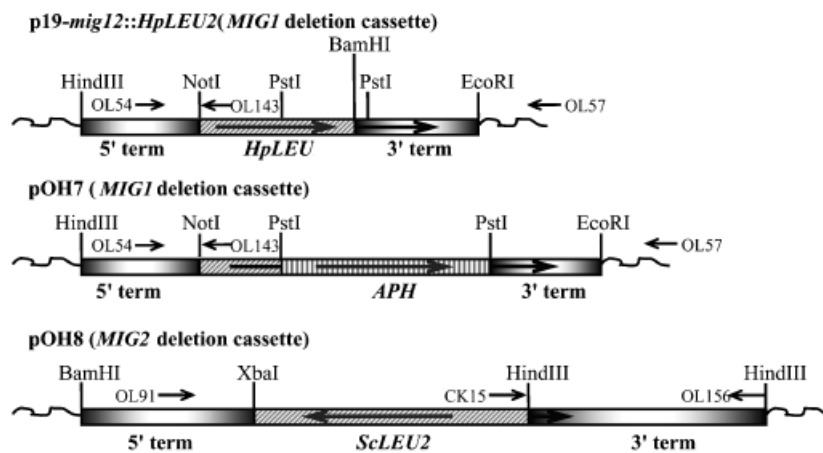
A *HpMIG1* (GenBank accession number EF523343) deletion cassette was constructed as follows: a 0.93-kb fragment containing sequences of the 3'-flanking region of *HpMIG1* was amplified by PCR using genomic DNA of *H. polymorpha* as a template and the primers Mi12Cf and Mi12Cr carrying restriction sites for EcoRI and BamHI (Table 1). The 3'-flanking fragment was inserted into the EcoRI/BamHI-digested plasmid pBSKT (Lombo *et al.*, 1997). Next, a 0.99-kb fragment consisting of the 5'-flanking region of *HpMIG1* was amplified by PCR with the primers Mi12Nf and Mi12Nr (Table 1) carrying restriction sites for HindIII and NotI, respectively. The 5'-flanking PCR fragment of *HpMIG1* and BamHI/NotI-digested *HpLEU2* fragment were cloned into the BamHI/NotI-digested plasmid pBSKT-3'*MIG1*, thus generating a deletion cassette for *HpMIG1* (plasmid p19-mig12::HpLEU2). The derivative plasmid pOH7 was constructed by replacing the PstI fragment of *HpLEU2* on p19-mig12::HpLEU2 with the PstI fragment harbouring the *APH* gene expressed under the constitutive promoter of glyceraldehyde-3-phosphate dehydrogenase from plasmid pGLG578 (Sohn *et al.*, 1999) (Fig. 1).

The  $\Delta$ *mig1* and  $\Delta$ *mig1 leu1-1* strains were isolated by transforming recipient wild-type strain NCYC495 *leu1-1* with deletion cassettes for *HpMIG1* from p19-mig12::HpLEU2 (EcoRI-HindIII fragment comprising *HpLEU2* flanked by *HpMIG1* 5'- and 3'-sequences) and pOH7 (Eco32I/PaeI fragment of pOH7 comprising *APH* flanked by the same *HpMIG1* sequences), thereby replacing the region of *HpMIG1* either with *HpLEU2* or with *APH*. Prototrophic or G418-resistant transformants were isolated and analysed as detailed below.

A *HpMIG2* (GenBank accession number EF523344) deletion cassette was constructed as follows: a 1.97-kb fragment containing sequences of the 3'-flanking region of *HpMIG2* was amplified by PCR using genomic DNA of *H. polymorpha* NCYC495 *leu1-1* as a template and the primers OL155 and OL156 carrying restriction sites for HindIII (Table 1, Fig. 1). The 3'-flanking fragment was inserted into the HindIII-digested plasmid pYT1 (Tan *et al.*, 1995), carrying the *ScLEU2* gene as a selection marker, to create pYT1-3'*MIG2*. Next, a 1.39-kb fragment consisting of the 5'-flanking region of *HpMIG2* was amplified by PCR with the primers OL153 and OL154 carrying restriction sites for BamHI and XbaI, respectively. The 5'-flanking PCR fragment of *HpMIG2* was cloned into the BamHI/XbaI-digested plasmid pYT1-3'*MIG2*, to create pOH8, generating a deletion cassette for *HpMIG2*.

The plasmid pOH8 was used as a template for PCR with primers OL153 and OL156, producing a 5.56-kb fragment comprising *ScLEU2* flanked by *MIG2* 5'- and 3'-sequences. The deletion cassette was transformed into NCYC495*leu1-1*, and prototrophic transformants selected.

Total genomic DNA isolated from potential  $\Delta$ *mig1* knock-out strains (p19-mig12::HpLEU2 and pOH7 transformants) was used as a template for PCR and Southern blot analyses. Primer pairs OL54/OL55, OL54/OL57 and OL54/OL143 (Table 1, Fig. 1) were used to determine the presence



**Fig. 1.** Scheme of construction of *Hansenula polymorpha*  $\Delta mig1$  and  $\Delta mig2$  deletion strains. The *HpMIG1*, *HpMIG2*, *HpLEU2*, *ScLEU2* and *APH* genes and directions of transcription are indicated by arrows in plasmids p19-mig12::*HpLEU2*, pOH7 and pOH8. p19-mig12::*HpLEU2* consists of a 943-bp fragment containing the 5' uncoding region of the *HpMIG1* gene (HindIII–NotI section), and a 991-bp fragment carrying the 3' uncoding region and short terminal part of the *HpMIG1* ORF (BamHI–EcoRI section). pOH7 consists of a 943-bp fragment containing the 5' uncoding region of the *HpMIG1* gene (HindIII–NotI section), and a 897-bp fragment carrying the 3' uncoding region and short terminal part of the *HpMIG1* ORF (PstI–EcoRI section). pOH8 consists of a 1.39-kb fragment containing the 5' uncoding region of the *HpMIG2* gene (BamHI–XbaI section), and a 1.9-kb fragment carrying the 3' uncoding region and short terminal part of the *HpMIG2* ORF (HindIII–HindIII section). Genomic DNA of the *H. polymorpha* that flank deletion cassettes upon integration into genome is represented by wavy line. Names and sequence position of the primers used in plasmid construction and PCR analysis of deletion mutants are indicated.

of corresponding knock-out cassettes in  $\Delta mig1$  and  $\Delta mig1 leu1-1$  genomic DNA. PCR analysis (data not shown) indicated the presence of expected bands in the tested strains: a 0.45-kb band generated by OL54/OL55, a 2.24-kb band (OL54/OL57), and the absence of the band (OL54/OL143) in the control strain NCYC495*leu1-1*. The 2.91-kb band (for  $\Delta mig1$ ) and 3.9-kb band (for  $\Delta mig1 leu1-1$ ) generated by OL54/OL57 and the 0.5-kb band generated by OL54/OL143 were present in knock-out strains. The pair of primers OL54/OL55 did not generate a band in the  $\Delta mig1$  and  $\Delta mig1 leu1-1$  strains.

Primer pairs OL91/OL92 and CK15/OL156 (Table 1, Fig. 1) were used for PCR analysis of the  $\Delta mig2$  strain. The 1.2-kb band generated by OL91/OL92 from genomic DNA of NCYC495*leu1-1* was absent in the deletion strain. The pair of primers CK15/OL156 was used for indication of a correctly targeted chromosomal integration of *mig2::HpLEU2* fragment. The 2.5-kb band was obtained with PCR for  $\Delta mig2$  and was absent in the control strain NCYC495*leu1-1*. Proper integration of the single *HpMIG1* and *HpMIG2* disruption cassettes was confirmed by Southern blot analysis (data not shown).

The identified  $\Delta mig1$  was used in all biochemical and physiological experiments in this study.  $\Delta mig1 leu1-1$  was used for construction of the  $\Delta mig1 \Delta mig2$  double-deletion mutant. The  $\Delta mig1 leu1-1$  and  $\Delta mig2$  strains were crossed and diploid hybrids were selected on YNS medium supplemented with G418 ( $1.0 \text{ g L}^{-1}$ ) without leucine. After sporulation, the  $\Delta mig1 \Delta mig2$  double-deletion mutant was selected among spore progeny on the same medium, and

confirmed for the presence of *HpMIG1* and *HpMIG2* deletion cassettes as detailed above for single-deletion mutants. Mating and sporulation techniques were performed using established procedures (Gleeson & Sudbery, 1988).

### Biochemical and ultrastructural analyses

Preparation of crude cell extracts, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analyses and preparation of cells for electron microscopy and immunocytochemistry were carried out as described in Waterham *et al.* (1994).

## Results

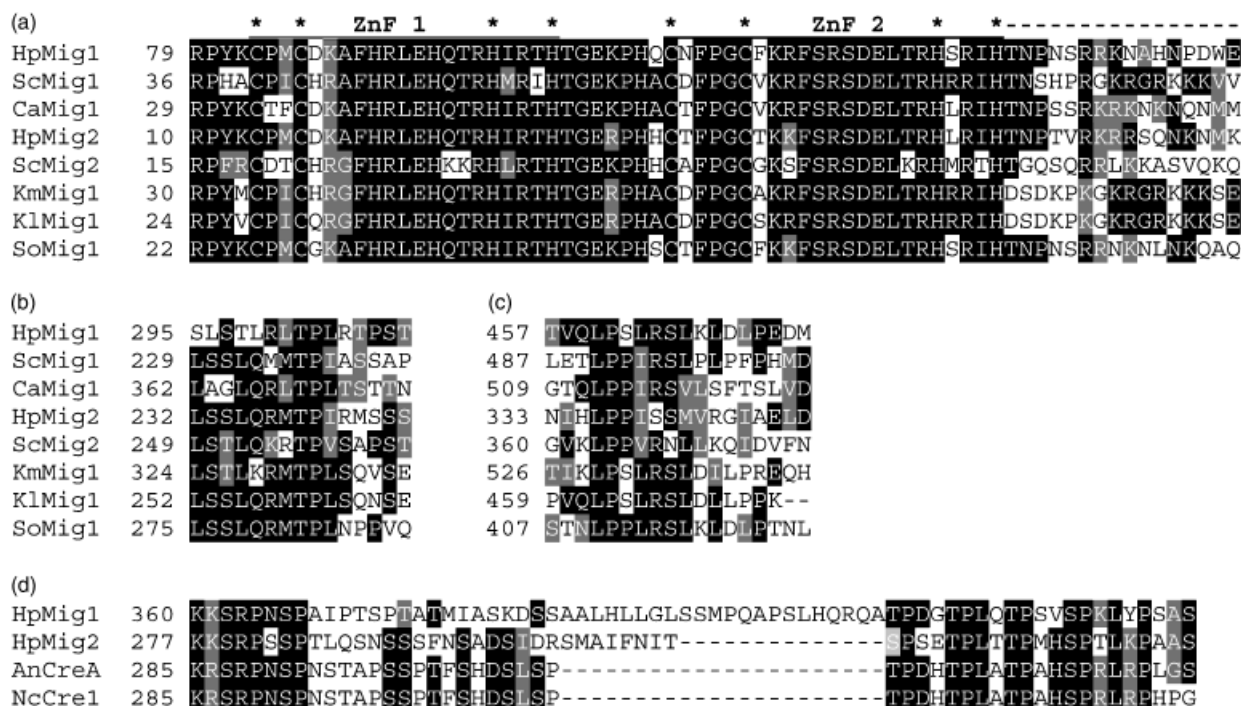
### Identification of *H. polymorpha* homologues of the *S. cerevisiae* Mig1 transcriptional repressor

A search in the *H. polymorpha* genome database (Ramezani-Rad *et al.*, 2003) revealed the presence of two putative homologues of *S. cerevisiae* Mig1 (ScMig1) C2H2 zinc-finger (ZnF) transcriptional repressor (Nehlin & Ronne, 1990). Similar results were obtained after probing the genome database with full-length ScMig1, or its N-terminal ZnF DNA-binding domain, which is highly conserved in all yeast Mig1 homologues (Ostling *et al.*, 1996; Cassart *et al.*, 1997; Zaragoza *et al.*, 2000; Carmona *et al.*, 2002), or the full-length sequence of the related *S. cerevisiae* Mig2 protein (Lutfiyya & Johnston, 1996). As expected, the two identified *H. polymorpha* Mig1 homologues, designated as *HpMig1* and *HpMig2*, exhibit limited overall homology to other yeast Mig proteins:

Despite the low sequence conservation aside the ZnF region, yeast Mig homologues possess other semi-conserved regions (Cassart *et al.*, 1997), present also in HpMig1 and HpMig2 (Fig. 2b and c), i.e. the so-called C-terminal 'effector domain' (Ostling *et al.*, 1996). In addition, the

### Isolation and physiological characterization of the *H. polymorpha* $\Delta mig1$ , $\Delta mig2$ and $\Delta mig1 \Delta mig2$ double-mutant strains

To analyse the physiological significance of the HpMig1 and HpMig2 repressors, we constructed *H. polymorpha* deletion strains in the corresponding genes by gene replacement (Fig. 1; for details see ‘Materials and methods’). Several  $\Delta mig1$  and  $\Delta mig2$  null mutants were isolated and all were identical with respect to their phenotype (see below). Because



**Fig. 2.** Multiple alignment of conserved regions of HpMig1 and HpMig2 proteins with yeast Mig1 and fungal CreA homologues. (a) N-terminal zinc-finger region. Two zinc-finger domains (ZnF) are overlined. Cysteine and histidine residues involved in ZnF domains are indicated with asterisks. Adjacent to ZnF basic region is overlined. (b) Highly conserved region of yeast Mig proteins. (c) Putative C-terminal effector domain. (d) Highly conserved region between *H. polymorpha* Mig and fungal CreA repressors. Species abbreviations are: Hp, *Hansenula polymorpha*; Sc, *Saccharomyces cerevisiae*; Ca, *Candida albicans*; Km, *Kluyveromyces marxianus*; Kl, *Kluyveromyces lactis*; So, *Schwanniomyces occidentalis*; An, *Aspergillus nidulans*; Nc, *Neurospora crassa*. GenPept accession numbers for proteins used in the alignments: SCY51 – P27705; ScMig2 – P53035; CaMig1 – Q9Y7G2; KmMig1 – P52288; KIMig1 – P50898; SoMig1 – CAD10675; AnCreA – AAR02858; NcCre1 – O59958.

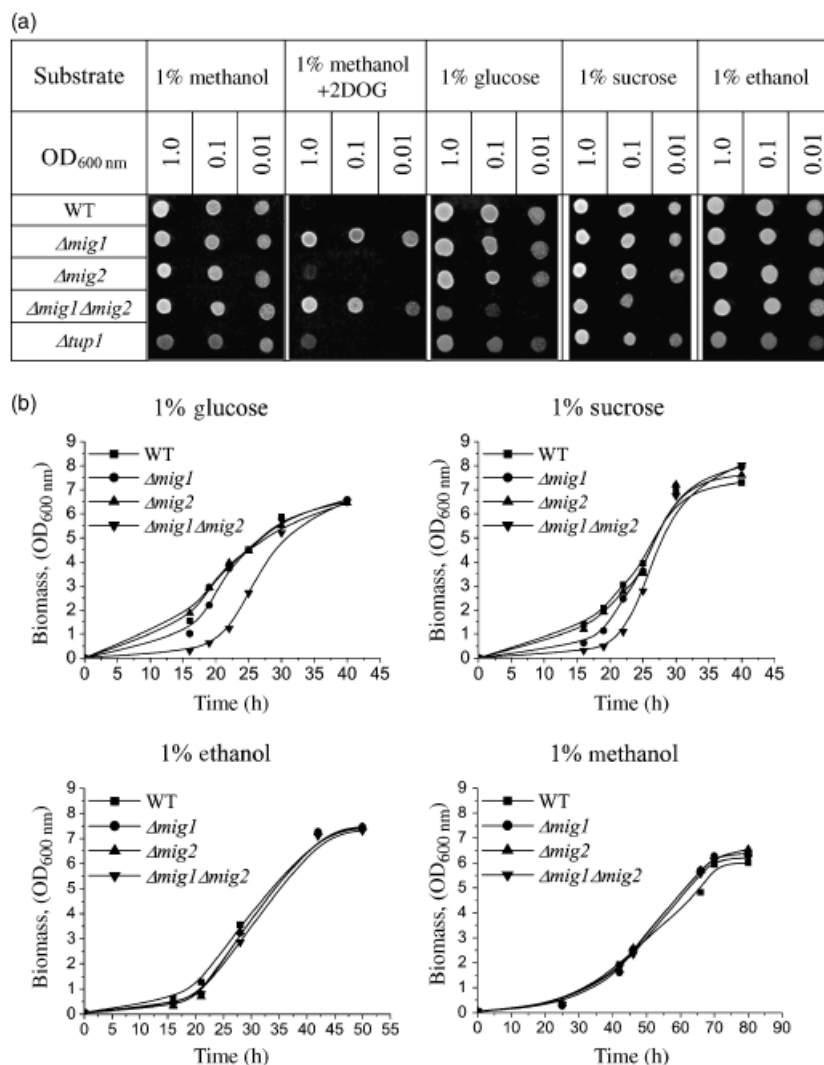
HpMig1 and HpMig2 may have a redundant function in transcriptional regulation as observed in *S. cerevisiae* (Lutfiyya *et al.*, 1998), a double-deletion  $\Delta mig1\Delta mig2$  mutant strain was isolated from spore progeny of the diploid, resulting from crossing  $\Delta mig1$  and  $\Delta mig2$  null mutants.

We observed that  $\Delta mig1$  and  $\Delta mig2$  mutants did not differ from the wild-type strain in growth on solid media supplemented with various carbon sources, namely glucose, sucrose (glycolytic substrates), ethanol and methanol, whereas the double-deletion mutant exhibited retarded growth on sugar substrates (Fig. 3a). The mutants behaved similarly on plates with low sugar concentrations (0.1% w/v) (not shown). However,  $\Delta mig1$  and  $\Delta mig1\Delta mig2$  mutants could grow on methanol plates in the presence of 2-deoxyglucose, suggesting an impairment of glucose repression. The *H. polymorpha*  $\Delta tup1$  mutant, which is not disturbed for glucose repression (Oliveira *et al.*, 2003), was used as a control.

Detailed analysis of growth kinetics in liquid cultures confirmed that *mig* mutants exhibit a wild-type growth rate on methanol and ethanol. When grown on sugar substrates, the lag phase in  $\Delta mig1$  and double  $\Delta mig1\Delta mig2$  mutants was extended, but growth was not affected in the exponential phase (Fig. 3b). This observation suggests that HpMig1 and HpMig2 may be involved in a transient transcriptional response upon glucose adaptation.

### Assessment of the role of HpMig1, HpMig2 and HpTup1 in catabolite repression

To gain further insight into HpMig1, HpMig2 and HpTup1 function in repression triggered by rich carbon sources, we assayed AO protein levels in the mutants. A very moderate impairment of AO repression was observed for  $\Delta mig1$ ,  $\Delta mig1\Delta mig2$  and  $\Delta tup1$  mutants incubated with low (0.1%) or high (1%) concentrations of glucose (Fig. 4). Pre-



**Fig. 3.** Growth properties of *Hansenula polymorpha mig* mutants on various carbon sources. (a) Equal volumes of YPS pre-grown cell suspensions were loaded in three dilutions on agarized media. Carbon substrate abbreviations are: Mth, methanol; Glc, glucose; 2-DOG, 2-deoxyglucose; Sucr, sucrose; Eth, ethanol. Cells were incubated for 1 day on sugar substrates, 2 days on methanol or ethanol, and 3 days on 2-DOG-containing medium. (b) Kinetics of growth in the liquid culture. Cells of the indicated *H. polymorpha* strains were pregrown on rich YPS medium and shifted to the mineral media with different carbon sources. OD<sub>600 nm</sub> was measured at the indicated time points after the shift.

viously described by us  $\Delta gcr1$  mutant strongly affected in glucose repression and constitutively synthesizing AO in glucose medium (Stasyk *et al.*, 2004) served as a positive control. Interestingly, when methanol was added to glucose medium, the defect in repression in  $\Delta mig1$  and  $\Delta mig1\Delta mig2$ , but not in  $\Delta tup1$ , became more pronounced (Fig. 4). This suggests that certain transcriptional induction components are induced for AO synthesis in *mig1* cells and may explain the ability of mutants to grow on methanol in the presence of 2-deoxyglucose (Fig. 3A). However, the AO protein level in the double  $\Delta mig1\Delta mig2$  mutant on carbon source mixture was considerably lower relative to glucose-grown  $\Delta gcr1$  cells or methanol-grown wild-type cells (Fig. 4 and legend).

Similarly, AO synthesis in  $\Delta mig1$ ,  $\Delta mig2$ ,  $\Delta mig1\Delta mig2$ , and  $\Delta tup1$  cells was only weakly derepressed in cells exposed to sucrose or ethanol (Fig. 4), suggesting a limited effect of the analysed mutations on the repression pathway(s) in *H. polymorpha*. We excluded peroxisome degradation as a principal factor influencing AO protein level in the analysed mutants grown on multicarbon substrates, as pexophagy is blocked (in *tup1*) or impaired in these strains (see next section).

### ***Hansenula polymorpha* Mig1 homologues interfere with pexophagy**

*Hansenula polymorpha* HpTup1 is the only transcriptional repressor shown so far to be essential for macropexophagy (Leao-Helder *et al.*, 2004). We were interested in elucidating whether its possible partners in transcriptional regulation, HpMig1 and HpMig2, also affect this process. In AO activity plate assays, upon a shift of methanol-grown cells to glucose or ethanol, we observed that  $\Delta mig1$  and  $\Delta mig1\Delta mig2$ ,

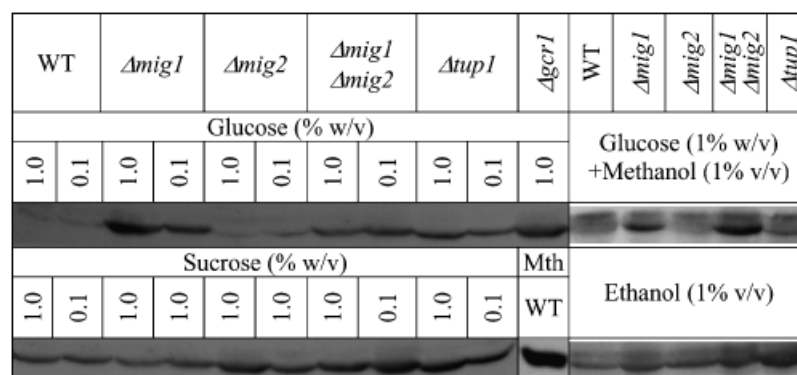
but not  $\Delta mig2$ , cells exhibit enhanced residual AO activities relative to wild-type controls. However, the defect in pexophagy in  $\Delta mig1$  was less pronounced relative to  $\Delta tup1$  cells (not shown). Similarly to the case for  $\Delta tup1$ , no *mig* mutant was significantly affected in general autophagy upon nitrogen starvation, as judged by means of the Phloxine-B plate test (Tsukada & Ohsumi, 1993) (not shown).

Detailed time-course analysis of pexophagy by following AO protein level revealed that the rate of AO degradation differed between the wild-type strain and *mig* mutants when methanol-grown cells were exposed to glucose or ethanol (Fig. 5a). However, Western blots demonstrated that, also during the growth of  $\Delta mig1\Delta mig2$  cells on methanol, minor AO degradation bands could be observed that were absent in wild-type controls (Fig. 5b), indicating that peroxisomes are degraded and that pexophagy is not completely blocked under these conditions in  $\Delta mig1\Delta mig2$  cells.

Electron microscopy of methanol-grown  $\Delta mig1\Delta mig2$  cells exposed to ethanol revealed that peroxisomes indeed undergo degradation and that AO protein can be detected in vacuolar lumen (Fig. 6c). Unexpectedly, whereas in wild-type controls organelles were degraded via macropexophagy (not shown), in  $\Delta mig1\Delta mig2$  cells the sequestration of organelles to be degraded was rarely observed (Fig. 6b). Instead, the morphological characteristics of micropexophagy, i.e. vacuolar protrusions surrounding peroxisome clusters, were prominent (Fig. 6a).

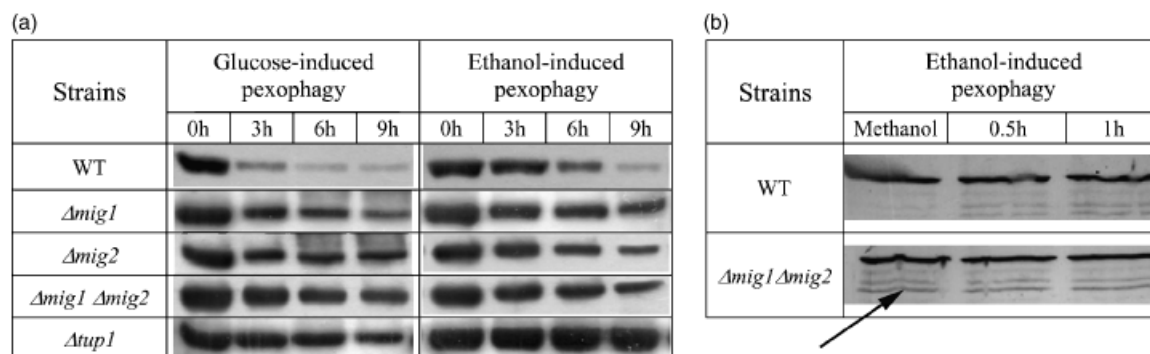
### **Discussion**

This paper describes the role of *H. polymorpha* transcriptional repressors in catabolite repression and peroxisome

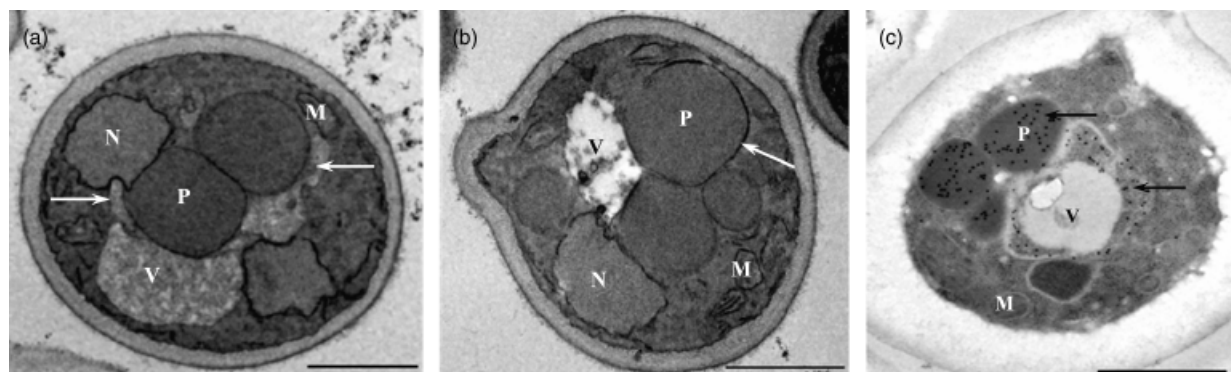


**Fig. 4.** Analysis of AO synthesis upon incubation of wild-type and mutant strains on various carbon sources. Cells of the indicated *H. polymorpha* strains were pre-grown in the rich YPS medium and shifted to mineral media supplemented with various carbon sources (concentrations are indicated), and then incubated for 6 h. One hundred micrograms of glucose-, sucrose- and ethanol-grown  $\Delta mig1$ ,  $\Delta mig2$ ,  $\Delta mig1\Delta mig2$ ,  $\Delta tup1$  and 10  $\mu$ g of glucose-grown  $\Delta gcr1$  TCA extracts of cultures were loaded per lane. Fifty micrograms of methanol-with-glucose-grown cultures were loaded per lane. Five micrograms of methanol-grown wild type culture was loaded per lane to serve as a control [Mth=1% methanol (v/v)]. AO protein was visualized by Western blotting with anti-AO bodies.





**Fig. 5.** Kinetic analysis of pexophagy. Cells of the indicated strains were grown on methanol medium and shifted to glucose and ethanol media to induce selective peroxisome degradation. Samples were taken at the indicated time points after the shift. Equal volumes of cultures were loaded per lane and analysed for the presence of AO protein by Western blotting using anti-AO bodies. (a) Kinetics of AO degradation induced by glucose or ethanol. (b) First stages of AO degradation induced by ethanol in wild-type and  $\Delta mig1 \Delta mig2$  strains. Notice the appearance of AO degradation products in extracts of  $\Delta mig1 \Delta mig2$  cells incubated in methanol before the shift (arrow).



**Fig. 6.** *Hansenula polymorpha*  $\Delta mig1 \Delta mig2$  cells are disturbed in macropexophagy but not in microautophagy. Methanol-grown  $\Delta mig1 \Delta mig2$  cells were exposed to ethanol for 1 h. (a) Peroxisomes are surrounded by vacuolar protrusions, indicative of microautophagy (arrows). (b) A peroxisome is sequestered by additional membrane structures. However, sequestration is not completed (arrow). (c) Immunolabelling reveals that, in methanol-grown  $\Delta mig1 \Delta mig2$  cells exposed to ethanol, AO protein is localized both to peroxisomes and to the vacuole, suggesting functional organelle degradation (arrows). M, mitochondrion; V, vacuole; P, peroxisome; N, nucleus. The bar represents 1  $\mu$ m.

degradation (catabolite inactivation) induced by rich carbon sources. We demonstrated that the repression pathway for peroxisomal AO in this yeast apparently only partially relies on the two Mig1 homologues, irrespective of the carbon source (glucose, sucrose or ethanol). However, addition of the inducer methanol elevates the AO protein level in  $\Delta mig1 \Delta mig2$  mutant incubated with glucose (Fig. 4), suggesting that this mutant exhibits a so-called 'inducible' phenotype (Sibirny *et al.*, 1988).

In *S. cerevisiae*, Mig1 is regulated by glucose (Ostling & Ronne, 1998) and facilitates binding of the general repressor complex Tup1-Ssn6 to the promoters of glucose-repressible genes (Gancedo, 1998). However, Tup1 is involved in pleiotropic functions through interaction with specific DNA-binding proteins for each functionally related set of

genes (reviewed in Malave & Dent, 2006), while the Mig1-dependent regulation is thought to be more specific, with a limited set of the target genes that include those repressed by glucose (Murad *et al.*, 2001). Therefore, our results are in line with the previous observation that HpTup1 is not required for glucose repression of peroxisomal enzymes (Oliveira *et al.*, 2003), and suggest that, at least for alcohol oxidase, operation of the classical *S. cerevisiae* pathway with Mig1/2-mediated Tup1-Ssn6 binding to the repressible promoter is unlikely. However, the redundant function of these repressors cannot be excluded, and the effect of combining all three mutations in one strain would be interesting to examine. An alternative explanation is the involvement of other still-unknown repressor(s). Therefore, further studies are required to obtain conclusive results on

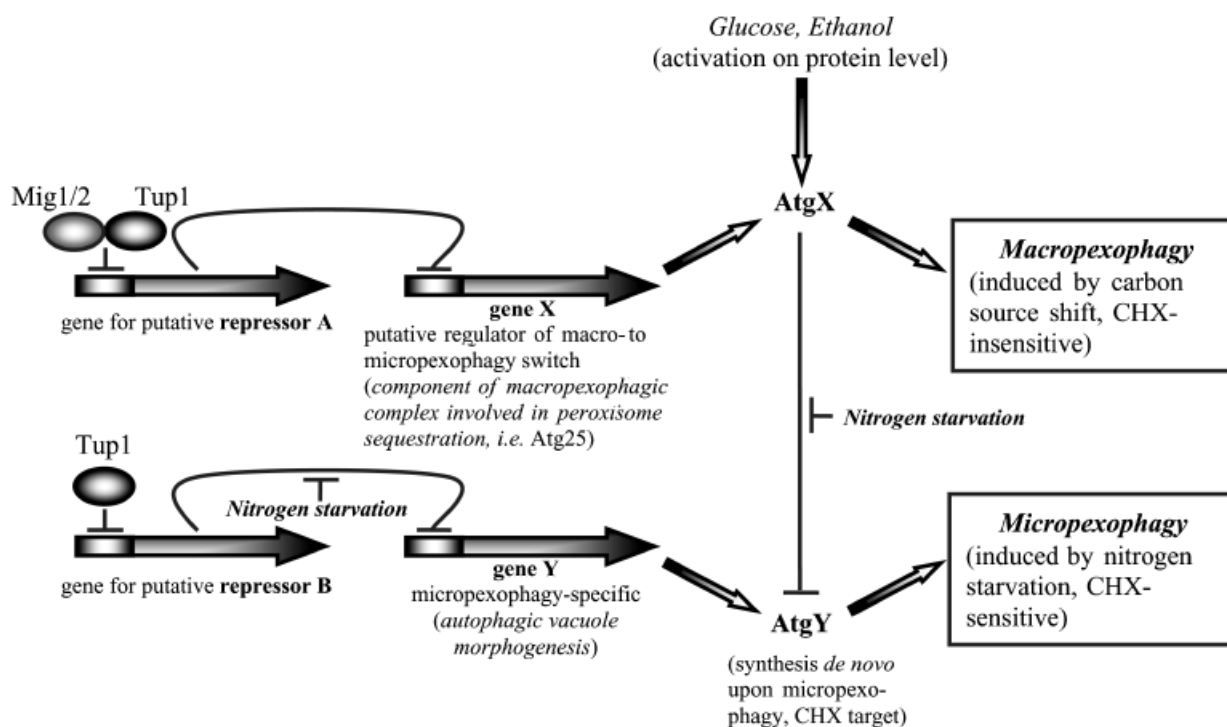
whether the effect of *mig1/2* and *tup1* mutations on catabolite repression in response to different carbon sources is direct or indirect. It also has to be investigated whether the analysed mutations interfere with the regulation of other glucose-repressible genes in *H. polymorpha*, for instance maltase, the regulation of which has been suggested to be Mig-dependent (Alamae *et al.*, 2003).

The pleiotropic function of Tup1 in yeast was recently extended to regulation of macropexophagy (Leao-Helder *et al.*, 2004). The finding that micropexophagy in *H. polymorpha* (Monastyrska *et al.*, 2005) and *P. pastoris* (Tuttle & Dunn, 1995) is sensitive to cycloheximide suggests that it is dependent on protein synthesis, and, consequently, on transcriptional regulation.

Our data suggest that the effect of Tup1 deletion on pexophagy is not mimicked by a similar deficiency in HpMig homologues. In *H. polymorpha* *tup1* mutant, macropexophagy is completely blocked, whereas micropexophagy occurs in the wild-type manner, i.e. only upon nitrogen starvation but not upon a shift of the carbon source (Leao-

Helder *et al.*, 2004). In the  $\Delta mig1\Delta mig2$  deletion strain, macropexophagy may also be impaired, whereas micropexophagy seems to be constitutively induced. This resembles the phenotype of *H. polymorpha*  $\Delta atg25$  mutant, which also displayed constitutive microautophagy in conjunction with a block in macropexophagy (Monastyrska *et al.*, 2005). This apparent similarity of the phenotypes of *H. polymorpha*  $\Delta mig1\Delta mig2$  and  $\Delta atg25$  mutants suggests that certain macropexophagy-specific components may be down-regulated in the absence of Mig repressors, and, hence, Mig repressors may be positive regulators of macropexophagy. Further research to elucidate the details of these effects is required, including the use of microarray approaches.

With regard to the control of micropexophagy, Mig1 homologues and Tup1 seem to have distinct roles depending on environmental conditions. Mig1 proteins may be negative regulators of micropexophagy, whereas Tup1 acts as a positive regulator, but only upon a shift in the carbon source and not upon nitrogen depletion conditions. A tentative model explaining such regulations is presented in Fig. 7.



**Fig. 7.** Tentative model of the involvement of HpMig- and HpTup1-mediated transcriptional repression in the regulation of pexophagy. We suggest that certain components of macropexophagy-specific machinery (such as HpAtg25) may be negative regulators of micropexophagy. Mig1/2 and Tup1 are indirect positive regulators of such hypothetical proteins (AtgX), which are activated posttranslationally by the effectors of macropexophagy, glucose and ethanol, and inhibit the hypothetical micropexophagic component AtgY. AtgX level in the absence of either Mig1/2 or Tup1 is decreased. Nitrogen starvation confers positive regulation of AtgY by inducing its expression and repressing macropexophagy at the protein level. In the absence of Tup1, but not Mig1/2, micropexophagy occurs only upon nitrogen starvation. We suggest that AtgY may be involved in the development of microautophagic vacuoles. CHX, cycloheximide.

## Acknowledgements

The authors gratefully acknowledge Rhein Biotech GmbH (Duesseldorf, Germany) for providing access to the *H. polymorpha* genome database. This work was partly supported by a grant from the Korean Ministry of Science and Technology (Microbial Genomics and Applications Research and Development Program).

## References

- Alamae T, Parn P, Viigand K & Karp H (2003) Regulation of the *Hansenula polymorpha* maltase gene promoter in *H. polymorpha* and *Saccharomyces cerevisiae*. *FEMS Yeast Res* **4**: 165–173.
- Carmona TA, Barrado P, Jiménez A & Lobato MF (2002) Molecular and functional analysis of a MIG1 homologue from the yeast *Schwanniomyces occidentalis*. *Yeast* **19**: 459–465.
- Cassart JP, Ostling J, Ronne H & Vandenhaute J (1997) Comparative analysis in three fungi reveals structurally and functionally conserved regions in the Mig1 repressor. *Mol Gen Genet* **255**: 9–18.
- Combet C, Blanchet C, Geourjon C & Deléage G (2000) NPS@: network protein sequence analysis. *TIBS* **25**: 147–150.
- Corpet F (1988) Multiple sequence alignment with hierarchical clustering. *Nucl Acids Res* **16**: 10881–10890.
- Dunn WA Jr, Cregg JM, Kiel JA, van der Klei IJ, Oku M, Sakai Y, Sibirny AA, Stasyk OV & Veenhuis M (2005) Pexophagy: the selective autophagy of peroxisomes. *Autophagy* **1**: 75–83.
- Faber KN, Haima P, Harder W, Veenhuis M & Ab G (1994) Highly-efficient electrotransformation of the yeast *Hansenula polymorpha*. *Curr Genet* **25**: 305–310.
- Gancedo JM (1998) Yeast carbon catabolite repression. *Microbiol Mol Biol Rev* **62**: 334–361.
- Gleeson MA & Sudbery PE (1988) Genetic analysis in the methylotrophic yeast *Hansenula polymorpha*. *Yeast* **4**: 293–303.
- Johnston M & Kim JH (2005) Glucose as a hormone: receptor-mediated glucose sensing in the yeast *Saccharomyces cerevisiae*. *Biochem Soc Trans* **33**: 247–252.
- Komduur JA, Bellu AR, Knoops K, van der Klei IJ & Veenhuis M (2004) Cold-inducible selective degradation of peroxisomes in *Hansenula polymorpha*. *FEMS Yeast Res* **5**: 281–285.
- Kramarenko T, Karp H, Jarviste A & Alamae T (2000) Sugar repression in the methylotrophic yeast *Hansenula polymorpha* studied by using hexokinase-negative, glucokinase-negative and double kinase-negative mutants. *Folia Microbiol (Praha)* **45**: 521–529.
- Krasovska OS, Stasyk OG, Nahorny VO, Stasyk OV, Granovski N, Kordium VA, Vozianov OF & Sibirny AA (2007) Glucose-induced production of recombinant proteins in *Hansenula polymorpha* mutants deficient in catabolite repression. *Biotechnol Bioeng* **97**: 858–870.
- Leao-Helder AN, Krikken AM, Lunenborg MG, Kiel JA, Veenhuis M & van der Klei IJ (2004) *Hansenula polymorpha* Tup1p is important for peroxisome degradation. *FEMS Yeast Res* **4**: 789–794.
- Lombo F, Siems K, Brana AF, Mendez C, Bindseil K & Salas JA (1997) Cloning and insertional inactivation of *Streptomyces argillaceus* genes involved in the earliest steps of biosynthesis of the sugar moieties of the antitumor polyketide mithramycin. *J Bacteriol* **179**: 3354–3357.
- Lutfiyya LL & Johnston M (1996) Two zinc-finger-containing repressors are responsible for glucose repression of *SUC2* expression. *Mol Cell Biol* **16**: 4790–4797.
- Lutfiyya LL, Iyer VR, DeRisi J, DeVit MJ, Brown PO & Johnston M (1998) Characterization of three related glucose repressors and genes they regulate in *Saccharomyces cerevisiae*. *Genetics* **150**: 1377–1391.
- Malave TM & Dent SY (2006) Transcriptional repression by Tup1-Ssn6. *Biochem Cell Biol* **84**: 437–443.
- Meijer WH, van der Klei IJ, Veenhuis M & Kiel JA (2007) ATG genes involved in non-selective autophagy are conserved from yeast to man, but the selective Cvt and pexophagy pathways also require organism-specific genes. *Autophagy* **3**: 106–116.
- Monastyrska I & Klionsky DJ (2006) Autophagy in organelle homeostasis: peroxisome turnover. *Mol Aspects Med* **27**: 483–494.
- Monastyrska I, Sjollem K, van der Klei IJ, Kiel JA & Veenhuis M (2002) Microautophagy and macropexophagy may occur simultaneously in *Hansenula polymorpha*. *FEBS Lett* **568**: 135–138.
- Monastyrska I, Kiel JA, Krikken AM, Komduur JA, Veenhuis M & van der Klei IJ (2005) The *Hansenula polymorpha* ATG25 gene encodes a novel coiled-coil protein that is required for macropexophagy. *Autophagy* **1**: 92–100.
- Murad AM, d'Enfert C, Gaillardin C, Tournu H, Tekai F, Talibi D, Marechal D, Marchais V, Cottin J & Brown AJ (2001) Transcript profiling in *Candida albicans* reveals new cellular functions for the transcriptional repressors CaTup1, CaMig1 and CaNrg1. *Mol Microbiol* **42**: 981–993.
- Nehlin JO & Ronne H (1990) Yeast MIG1 repressor is related to the mammalian early growth response and Wilm's tumour finger proteins. *EMBO J* **9**: 2891–2898.
- Oliveira MA, Genu V, Salmazo AP, Carraro DM & Pereira GA (2003) The transcription factor Snf1p is involved in a Tup1p-independent manner in the glucose regulation of the major methanol metabolism genes of *Hansenula polymorpha*. *Genetics and Mol Biol* **26**: 521–528.
- Ostling J & Ronne H (1998) Negative control of the Mig1p repressor by Snf1p-dependent phosphorylation in the absence of glucose. *Eur J Biochem* **252**: 162–168.
- Ostling J, Carlberg M & Ronne H (1996) Functional domains in the Mig1 repressor. *Mol and Cell Biol* **16**: 753–761.
- Papamichos-Chronakis M, Gligoris T & Tzamarias D (2004) The Snf1 kinase controls glucose repression in yeast by modulating interactions between the Mig1 repressor and the Cyc8-Tup1 co-repressor. *EMBO Rep* **5**: 368–372.

- Pereira GG & Hollenberg CP (1996) Conserved regulation of the *Hansenula polymorpha* MOX promoter in *Saccharomyces cerevisiae* reveals insights in the transcriptional activation by Adr1p. *Eur J Biochem* **238**: 181–191.
- Ramezani-Rad M, Hollenberg CP, Lauber J, Wedler H, Griess E, Wagner C, Albermann K, Hani J, Piontek M, Dahlems U & Gellissen G (2003) The *Hansenula polymorpha* (strain CBS4732) genome sequencing and analysis. *FEMS Yeast Res* **4**: 207–215.
- Roggenkamp R, Janowicz Z, Stanikowski B & Hollenberg CP (1984) Biosynthesis and regulation of the peroxisomal methanol oxidase from the methylotrophic yeast *Hansenula polymorpha*. *Mol Gen Genet* **194**: 489–493.
- Ronne H (1995) Glucose repression in fungi. *Trends Genet* **11**: 12–17.
- Sakai Y, Oku M, van der Klei IJ & Kiel JA (2006) Pexophagy: autophagic degradation of peroxisomes. *Biochim Biophys Acta* **1763**: 1767–1775.
- Sambrook J, Fritsh EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sibirny AA, Titorenko VI, Gonchar MV, Ubiyovk VM, Ksheminskaya GP & Vitvitskaya OP (1988) Genetic control of methanol utilization in yeasts. *J Basic Microbiol* **28**: 293–319.
- Sohn JH, Choi ES, Kang HA, Rhee JS, Agaphonov MO, Ter-Avanesyan MD & Rhee SK (1999) A dominant selection system designed for copy-number-controlled gene integration in *Hansenula polymorpha* DL-1. *Appl Microbiol Biotechnol* **51**: 800–807.
- Stasyk OV, Stasyk OG, Komduur J, Veenhuis M, Cregg JM & Sibirny AA (2004) A hexose transporter homologue controls glucose repression in the methylotrophic yeast *Hansenula polymorpha*. *J Biol Chem* **279**: 8116–8125.
- Tan X, Waterham HR, Veenhuis V & Cregg JM (1995) The *Hansenula polymorpha* PER1 gene encodes a novel peroxisomal integral membrane protein involved in proliferation. *J Cell Biol* **128**: 307–319.
- Treitel MA, Kuchin S & Carlson M (1998) Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in *Saccharomyces cerevisiae*. *Mol Cell Biol* **18**: 6273–6280.
- Tsukada M & Ohsumi Y (1993) Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett* **333**: 169–174.
- Tuttle DL & Dunn WA Jr (1995) Divergent modes of autophagy in the methylotrophic yeast *Pichia pastoris*. *J Cell Sci* **108**: 25–35.
- Tuttle DL, Lewin AS & Dunn WA Jr (1993) Selective autophagy of peroxisomes in methylotrophic yeasts. *Eur J Cell Biol* **60**: 283–290.
- van der Klei IJ, Yurimoto H, Sakai Y & Veenhuis M (2006) The significance of peroxisomes in methanol metabolism in methylotrophic yeast. *Biochim Biophys Acta* **1763**: 1453–1462.
- van Dijk R, Faber KN, Kiel JA, Veenhuis M & van der Klei I (2000) The methylotrophic yeast *Hansenula polymorpha*: a versatile cell factory. *Enzyme Microb Technol* **26**: 793–800.
- Veenhuis M, Douma A, Harder W & Osumi M (1983a) Degradation and turnover of peroxisomes in the yeast *Hansenula polymorpha* induced by selective inactivation of peroxisomal enzymes. *Arch Microbiol* **134**: 193–203.
- Veenhuis M, Van Dijken JP & Harder W (1983b) The significance of peroxisomes in the metabolism of one-carbon compounds in yeasts. *Adv Microb Physiol* **24**: 1–82.
- Waterham HR, Titorenko VI, Haima P, Cregg JM, Harder W & Veenhuis M (1994) The *Hansenula polymorpha* PER1 gene is essential for peroxisome biogenesis and encodes a peroxisomal matrix protein with both carboxy and amino-terminal targeting signals. *J Cell Biol* **127**: 737–749.
- Zaragoza O, Rodriguez C & Gancedo C (2000) Isolation of the MIG1 gene from *Candida albicans* and effects of its disruption on catabolite repression. *J Bacteriol* **182**: 320–326.